European Journal of Pharmaceutical Sciences 101 (2017) 228–242

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Mixed micelles of lipoic acid-chitosan-poly(ethylene glycol) and distearoylphosphatidylethanolamine-poly(ethylene glycol) for tumor delivery

Zeeneh Elsaid [a,*], Kevin M.G. Taylor [a], Sanyogitta Puri [b], Cath A. Eberlein [b], Khuloud Al-Jamal [c], Jie Bai [c], Rebecca Klippstein [c], Julie Tzu-Wen Wang [c], Ben Forbes [c], Jasminder Chana [c], Satyanarayana Somavarapu [a,*]

[a] UCL School of Pharmacy, 29-39 Brunswick Square, London WC1N 1AX, United Kingdom
[b] AstraZeneca, Macclesfield, Cheshire East SK10 2NA, United Kingdom
[c] Kings College London, Franklin-Wilkins Building, Stamford Street, London SE1 8WA, United Kingdom

ARTICLE INFO

Article history:
Received 31 October 2016
Received in revised form 27 January 2017
Accepted 1 February 2017
Available online 3 February 2017

Keywords:
Mixed micelles
Cancer
Hydrophobic drug
Biodistribution
Uptake
Chitosan
DSPE-PEG

ABSTRACT

Many chemotherapeutics suffer from poor aqueous solubility and tissue selectivity. Distearoylphosphatidylethanolamine-poly(ethylene glycol) (DSPE-PEG) micelles are a promising formulation strategy for the delivery of hydrophobic anticancer drugs. However, storage and *in vivo* instability restrict their use. The aim of this study was to prepare mixed micelles, containing a novel polymer, lipoic acid-chitosan-poly(ethylene glycol) (LACPEG), and DSPE-PEG, to overcome these limitations and potentially increase cancer cell internalisation. Drug-loaded micelles were prepared with a model tyrosine kinase inhibitor and characterized for size, surface charge, stability, morphology, drug entrapment efficiency, cell viability (A549 and PC-9 cell lines), *in vivo* biodistribution, *ex vivo* tumor accumulation and cellular internalisation. Micelles of size 30–130 nm with entrapment efficiencies of 46–81% were prepared. LACPEG/DSPE-PEG mixed micelles showed greater interaction with the drug (condensing to half their size following entrapment), greater stability, and a safer profile *in vitro* compared to DSPE-PEG micelles. LACPEG/DSPE-PEG and DSPE-PEG micelles had similar entrapment efficiencies and *in vivo* tumor accumulation levels, but LACPEG/DSPE-PEG micelles showed higher tumor cell internalisation. Collectively, these findings suggest that LACPEG/DSPE-PEG mixed micelles provide a promising platform for tumor delivery of hydrophobic drugs.

1. Introduction

Chemotherapeutic agents act *via* various mechanisms to inhibit tumor growth, angiogenesis and metastasis, as well as to increase cancer cell apoptosis. Poor aqueous solubility and bioavailability are key limitations for several of these agents that are administered *via* the oral route. In the clinic, in order to achieve therapeutic concentrations at the target site, dose escalation of the drug is sometimes employed (Farhat and Houhou, 2013; Xue et al., 2015) which is, in turn, associated with increased toxicity. Furthermore, poor aqueous solubility also prevents their administration intravenously, a route which may be considered for patients incapable of taking the drug orally. Thus, it would be extremely beneficial to develop alternative delivery systems for these agents.

Polymeric micelles (<100 nm), comprising amphiphilic polymers, are emerging as promising drug delivery vehicles for poorly water-soluble drugs (Kim et al., 2010). Their structure presents a number of advantages; with their inner core aiding hydrophobic drug incorporation and the outer hydrophilic shell reducing particle aggregation and opsonisation (Torchilin, 2001), thereby hindering uptake by the reticuloendothelial system (Haag, 2004; Torchilin, 2007). The latter prolongs the circulation half-life, an effect which when considered with their small size, promotes passive accumulation at the tumor site as a result of the enhanced permeation and retention (EPR) effect (Maeda et al., 2013). These advantages for site-specific drug delivery could potentially improve the delivery of traditional and emerging chemotherapeutic drugs, which might otherwise have been abandoned due to insolubility and toxicity.

An increasing number of micelle formulations are under investigation in preclinical and clinical studies for the delivery of hydrophobic anticancer drugs. One such example is NK012, which has entered phase II clinical trials. This is a block copolymer of poly(ethylene glycol) (PEG) and polyglutamate conjugated to the drug, 7-ethyl-10-hydroxycampothecin (SN-38). This micellar system demonstrated promising results with an up to 5.8-fold higher IC50 values and lower clearance relative to free SN-38 (Burris et al., 2016; Koizumi et al.,

* Corresponding authors.
E-mail addresses: elsaid_a@hotmail.com (Z. Elsaid), s.somavarapu@ucl.ac.uk (S. Somavarapu).

http://dx.doi.org/10.1016/j.ejps.2017.02.001
0928-0987/

2006). Another example is Genexol-PM, which has entered phase II clinical trials for NSCLC, and comprises polymeric micelles composed of PEG and poly(D,L-lactic acid), and was shown to enhance the maximum tolerated dose and antitumor efficacy of paclitaxel (Kim et al., 2001). This micellar formulation has also reached the market in a number of places, including Korea and Europe (Pillai, 2014). Also, Afatinib within micelles, prepared from a polymer blend of MPEG-PCL/Mal-PEG-PCL, demonstrated higher cytotoxic activity and growth suppression in tumors obtained from colorectal cancer patients than compared to drug solution alone (Guan et al., 2014).

All of these micelles contain PEG, which provides steric hindrance, reducing aggregation and physically stabilizing the preparations. It also reduces clearance by the reticuloendothelial system, improving the drug/micelle circulation half-life and allowing it to reach the tumor site post intravenous injection (Alexis et al., 2008; Maeda et al., 2000; Maruyama, 2011). Micelles composed of PEG and phospholipids, such as Distearoylphosphatidylethanolamine-poly(ethylene glycol) (DSPE-PEG), have generated considerable interest due to their small size which promotes passive accumulation at the target site secondary to the EPR effect (Diao et al., 2011; Maeda, 2001; Matsumura and Maeda, 1986), prolonged circulation half-life (Maruyama et al., 1992), enhanced cellular uptake (Wang et al., 2012), reduced toxicity (Deol and Khuller, 1997) and high loading (of up to 95%) of hydrophobic anticancer drugs (Sezgin et al., 2006; Wang et al., 2012). However, storage and *in vivo* instability limit their use and hinder their translation into the clinic. Storage of DSPE-PEG micellar solutions for longer than 1 week, at 25 °C, results in carrier instability and precipitate formation (Johnsson et al., 2001). Additionally, following injection *in vivo*, DSPE-PEG micelle destabilization may occur due to significant dilution (critical micelle concentration is 0.5–1 μM (Ashok et al., 2004)), an outcome which is exacerbated by serum proteins, such as bovine serum albumin (BSA), which interact with the hydrophobic components of the micelles (Castelletto et al., 2007; Kastantin et al., 2010), resulting in the premature release of the encapsulated agents prior to the micelles reaching their target site. Previous studies have shown that the use of two or more amphiphilic polymers, to prepare mixed micelles, can overcome these limitations; micelles possess greater *in vivo* stability, as the hydrophobic components of the carrier are screened by the di-block mPEG component of the micelles, reducing its interaction with the BSA and efficiently preventing BSA adsorption onto the mixed micelles (Li et al., 2011). Mixed micelles have also shown greater storage stability, attributed to the increased interactions between the hydrophobic chains in the micellar core which stabilize the structure, or to specific polymer characteristics, such as PEG content (Gao et al., 2008; Li et al., 2011; Zheng et al., 2016). Mixed micelles also provide the added advantages of improved drug loading and cellular uptake, as demonstrated for a range of hydrophobic drugs (Attia et al., 2013; Butt et al., 2012; Cao et al., 2016; Kahook et al., 2010; Yang et al., 2010). Moreover, the FDA has Doxil®, an intravenously administered liposome formulation of the cytotoxic drug doxorubicin, with three lipid components, including DSPE-PEG.

A second novel polymer, lipoic acid-chitosan-poly(ethylene glycol) LACPEG, with desirable properties to overcome the stability limitations of conventional micelles, can be used with DSPE-PEG to form mixed micelles with potential to improve hydrophobic drug delivery. The design of LACPEG was informed by a number of factors. Lipoic acid has antioxidant and anti-inflammatory activities (Weerakody et al., 2008), which could help to reduce oxidative stress and inflammation, two crucial pathways involved in carcinogenesis (Reuter et al., 2010). It has also been shown to inhibit cell proliferation (by inhibiting the Akt pathway and up-regulating a cyclin-dependent kinase inhibitor) and growth (by inhibiting tyrosine phosphorylation, and hence activation of growth factor receptors), as well inducing the selective apoptosis of a number of cancer cell lines, such as colon and lung cancer, (by increasing caspase activity, increasing the uptake of oxidizable substrates into the mitochondria and changing the ratio of the apoptotic-related protein Bax/Bcl-2) (Choi et al., 2009; Dozio et al., 2010; Michikoshi et al., 2013; Na et al., 2009; Wenzel et al., 2005). These properties could help to augment those of the drug and result in a greater inhibition of tumor cell growth. Chitosan was selected for its mucoadhesive, antioxidant, antiangiogenic and wound-healing properties (Sogias et al., 2008; Xu et al., 2009; Yen et al., 2008), as well as its polycationic nature which may increase cellular uptake (with amino groups which could be ionized at acidic pH in the tumor environment).

Based on the above, the aim of this study was to prepare mixed micelles of LACPEG and DSPE-PEG and to analyse their efficacy in preclinical *in vitro* and *in vivo* studies for the delivery of a hydrophobic anticancer drug. A prototype EGFR tyrosine kinase inhibitor with the following characteristics was selected; molecular weight (Mw < 1 kDa), sparingly soluble or practically insoluble across the physiological pH range and Log P: 4.85. This is the first study which investigates the use of this novel polymer, LACPEG, with DSPE-PEG for the delivery of a hydrophobic anticancer drug.

2. Materials and Methods

2.1. Materials

The hydrophobic drug used is AstraZeneca proprietary (Log *P*: 4.85, pKa: 5.42 and 7.24). Chitosan oligosaccharide (Mw 3–5 kDa, degree of deacetylation, >90%, Kittolife Co., Seoul, Korea), 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-*n*-methoxy(polyethylene glycol)-2000 (DSPE-PEG, Mw 2 kDa, Lipoid GmBH, Germany) were used in this study. The following reagents, solvents and solutions were obtained from Sigma Aldrich (USA): poly(vinyl alcohol) (PVA, Mw 13–23 kDa, 87–89% hydrolyzed), lipoic acid, *N*-hydroxysuccinimide (NHS, 98%), dichloromethane (DCM, >99.8%, anhydrous), dimethylsulfoxide (DMSO, anhydrous, >99.9%), sodium acetate, RPMI-1640, L-glutamine 200 mM, 100× antibiotic-antimycotic solution, Dulbecco's phosphate buffered saline (DPBS) and Triton X-100. The following materials were purchased from Fisher (UK): sodium hydroxide, acetonitrile (ACN, HPLC grade) and ethyl acetate. The following materials were also used: *N*, *N*-dicyclohexylcarbodiimide (DCC, Alfa Aesar, USA), methoxy-PEG-*N*-hydroxy succinimide (mPEG-NHS, Mw 5.4 kDa, Advanced Polymer Materials, Canada), Opti-MEM® and TrypLE express and foetal bovine serum (FBS) (Gibco Invitrogen, USA), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium compound (MTS) and phenazine methosulfate (PMS) (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, USA) and PLGA (DL-lactide/glycolide ratio 85:15, Mw 149 kDa, Alkermes Medisorb, USA). Live/Dead® BacLight viability kit containing propidium iodide and Syto 9 dye was purchased from Life Technologies Corporation, USA. The following materials were purchased for *in vivo* studies: Advanced RPMI-1640 media, penicillin-streptomycin 100×, Trypsin-EDTA (1×) with phenol red Glutamax™ supplement, phosphate buffered saline PBS (10× and 1×, pH 7.4) were obtained from Gibco, Invitrogen (UK), plasma derived bovine serum (FBS) (First-Link, UK), pentobarbital sodium (Euthatal®, Merial, UK), Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) and carbocyanine perchlorate dye (DiI, Santa Cruz Biotechnology, USA). HPLC grade deionized water was used throughout, unless otherwise stated.

2.2. Synthesis of LACPEG

1 g lipoic acid (4.85 mmol) was dissolved in 20 mL DCM and reacted with NHS (560 mg, 4.87 mmol) and DCC (820 mg, 3.97 mmol), each dissolved in 10 mL of DCM. This reaction was carried out in the dark, under nitrogen at room temperature for 48 h. The solution was filtered (Whatman filter paper, 150 mm) to remove dicyclohexylurea crystals and rotary evaporated at 60 °C and 280 rpm (Heidolph, UK) to remove solvent. The resultant thin film was dissolved in 20 mL anhydrous DMSO. 1 g mPEG-NHS (0.19 mmol) dissolved in 10 mL anhydrous